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The Calibration by Electronic Means of Coulter Counter for the Determination of Absolute Volume of Particles*

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Summary

An electronic method is described for the calibration of a particle sizing apparatus based on the principle employed by Coulter counters, without any need for reference particles. A capillary factor, depending upon the dimensions of the orifice, has been obtained by reference measurements. Incorporating this capillary factor and the particle form factor, the absolute particle volumes can be deduced.

Zusammenfassung

Die Arbeit beschreibt eine elektronische Methode zur Eichung von Partikelvolumen-Meßgeräten nach dem Coulter-Prinzip ohne Verwendung von Referenzpartikeln bekannter Größe. Durch Referenzmessungen wird ein von den Dimensionen der Meßöffnung abhängiger Kapillarfaktor bestimmt. Die Einbeziehung dieses Kapillarfaktors in bekannte Berechnungsformeln gestattet bei Kenntnis des Partikelformfaktors eine Berechnung absoluter Volumina.

The measurement of volume distribution curves (VDC) using the principle employed by Coulter counters is achieving ever greater significance, especially in the field of hematology. Recently it was shown [*9*] that Coulter capillaries modified with a central beam attachment no longer exhibit VDC for particles having a Gaussian distribution which are skewed to the right; curves of this type are characteristic for the usual Coulter capillaries [*1,4,7,12,15*]. The modified capillaries provide better resolution and make the analysis of erythrocyte VDC easier [*19*].

For all measurements of absolute particle volumes a suitable calibration of the apparatus has been a problem, as well as a suitable calibration control between each measurement to eliminate influences from instrument drift or capillary contamination on the VDC. There are two distinct calibration methods for the sizing of absolute particle volumes:

1) The particle calibration

Native or fixed human or animal erythrocytes, polystyrene particles or pollen are used [*1–4,7,11–17*]. This method excludes possible sources of error in the measuring system only if the following conditions are met:

a) Volume fluctuations of the particles due to the methods and duration of storage or the suspension medium in which they are measured must be excluded. Neither

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native or glutaraldehyde-fixed erythrocytes nor pollen fulfill these conditions. Polystyrene particles are however well suited.

b) The calibration particles must have volumes comparable to those of the test samples to avoid error due to imprecisely calibrated apparatus. However, polystyrene particles with little dispersion are currently available in only a few size ranges.

2) The electronic calibration and the calculation of the absolute volumes by known equations [*5–8*].

Gutmann [*7*] gives the following equation for experimental convenience:

$$V = 10 \cdot \frac{R^4 \cdot x}{\rho \cdot D}$$

This reduces by transformation to the basic equation for electronic particle sizing:

$$\frac{V}{V_L} = \frac{\Delta Z}{Z} \quad [10] \quad \text{whereby}$$

V = particle volume in μm^3
V_L = volume of the capillary in μm^3 ($V_L = R^2 \pi l$, l = capillary length in μm)
R = radius of the capillary in μm
x = impulse height in Volts for which the volume is to be calculated
ρ = specific resistance of the suspending buffer in Ohms × cm
D = calibration factor in Volts/Ohm
Z = initial resistance of the capillary
ΔZ = change in the resistance of the capillary during the passage of the particle.

For both methods the calibration is only accurate when the particles are non-conductive in comparison to the suspending buffer. In addition, the measurements with both calibration methods have to be corrected for the form factor [*5,6,9,18*]. This takes into account that the particle volume is overestimated to a variable degree by electrical sizing depending on the particle form during passage through the capillary.

This paper reports the investigation into whether an electronic calibration together with the equation of Gutmann [*7*] leads to correct particle volumes for various capillaries, particles and settings of the measuring apparatus.

Methods

1. Mean volume of the polystyrene:

6-E Polystyrene particles DVB (Particle Information Service, Los Altos, Calif.) were used. They were washed twice in double distilled water and resuspended in double distilled water at a concentration of ca. 2.5 × 10^5 particles/mm^3 and 20° C. After thoroughly mixing the polystyrene suspension, 100 µl were removed with an Eppendorf pipette (Netheler and Hinz, Hamburg), weighed out to the nearest 0.1 mg on a watchglass, dried at 105° C and reweighed when cooled to room temperature. The difference of the two weights represented the weight of the evaporated water and simultaneously, because the density of water at 20° C is 1.0 (g/cm^3), of the evaporated volume. The total volume of the polystyrene particles was obtained from the difference between initial and evaporated volume.

The particle concentration in the suspension was measured in a 0.95% phosphate buffered NaCl solution, pH 7.4, at a dilution of 1 : 10000 with a Coulter-A particle counter using a 100 µm capillary with amplification 1, current 6 and threshold 6. The mean particle volume

in μm^3 was calculated from the ratio of total volume of the polystyrene particles to number of particles per 0.1 ml suspension. The microscopic examination of the polystyrene particle suspension revealed no signs of agglutination.

2. Mean cellular volume (MCV) of normal human and rat erythrocytes:

Freshly drawn blood from healthy donors and rat erythrocytes from female SPF-Sprague-Dawley-rats (Gassner, München-Ottobrunn), weighing approximately 100 g were used throughout the experiments. The blood hematocrit was measured after 15 min. centrifuging at 12000 × g using microhematocrit tubes of 75 mm length and 1.1 mm inner diameter (Clay-Adams, N. Y., N. Y.). The erythrocytes were counted in a 0.95% phosphate buffered NaCl solution, pH 7.4, at a dilution of 1 : 50000 using a Coulter A particle counter at amplification 1, current 8 and threshold 5. The mean erythrocyte volume in μm^3 was calculated from the ratio obtained by dividing microhematocrit by cell count/mm^3.

3. Electrical measurement of erythrocytes and 6-E polystyrene particles DVB:

Coulter capillaries with 70, 100 and 200 μm orifice diameters were modified by the central beam attachment described by Kachel et al [*9*]. The polystyrene particles or erythrocytes were suspended in a 0.95% phosphate buffered NaCl solution, pH 7.4, in a concentration such that the measuring speed did not exceed 250 particles/sec. Using an impulse height analyzer JFK [*10*] together with a 20 channel storage and recording unit UC (Rohde und Schwarz, Munich), the particle VDC were recorded at a temperature of 25° C. About 2×10^4 particles per VDC were measured. Currents between 0.07 and 1.0 mA together with appropriate impulse amplification were used with the different capillaries and particles.

4. The electronic calibration of the particle sizing apparatus:

a) The diameters of the 70, 100 and 200 μm capillaries were determined with a Zeiss UMM measuring microscope. They measured within ± 0.5 μm of the specifications given by the manufacturer. The capillary lengths were determined to 5 μm by turning the capillary 45° under the microscope, measuring the apparent orifice diameter and the apparent capillary length and calculating the true capillary length as a trigonometric function of the apparent one. The 70 μm ⌀ capillary was 35 μm long, the 100 μm ⌀ 71 μm long and the 200 μm ⌀ 126 μm long, the length to diameter ratios being 0.50, 0.71 and 0.63 respectively.

b) The specific resistance of the buffer solutions was determined at 25° C with a conductivity measuring cell (Philips PR 9513) and a resistance measuring bridge (Philips GM 4249/01).

c) The electronic calibration simulates the impulse that is generated as the particle passes through the capillary. An impulse generator as described by *Gutmann* [*7*] was used. In addition the resistance of the capillary was imitated by an Ohm-resistance joined in series with the impulse generator. The calibration factor D was determined as the impulse height in Volts produced by the impulse generator for a change of resistance of 1 Ohm. The current and amplification adjustment of the apparatus were the same as for the VDC, whose volume parameters were to be calculated.

5. Statistical treatment of the results:

For each series of experiments the mean and the standard deviation were calculated.

Results

1) Measurement of the mean particle volume by the reference methods:

a) 16 measurements of the gravimetrically determined mean volume of 6-E polystyrene particles DVB yielded a value of 551 ± 20 μm^3.

b) The mean volume of human and rat erythrocytes for 20 measurements of each gave values of 95 ± 6 μm^3 and 66 ± 4 μm^3.

2) Electronic measurement of particle volumes using the central beam method:

a) Due to the band width of 150 kHz of the amplifier the correct transfer of the impulse height is dependent on the resistance of the capillary and on the suction pressure which markedly influences the particle speed through the capillary and the impulse length. Especially at higher particle speeds a significant loss of

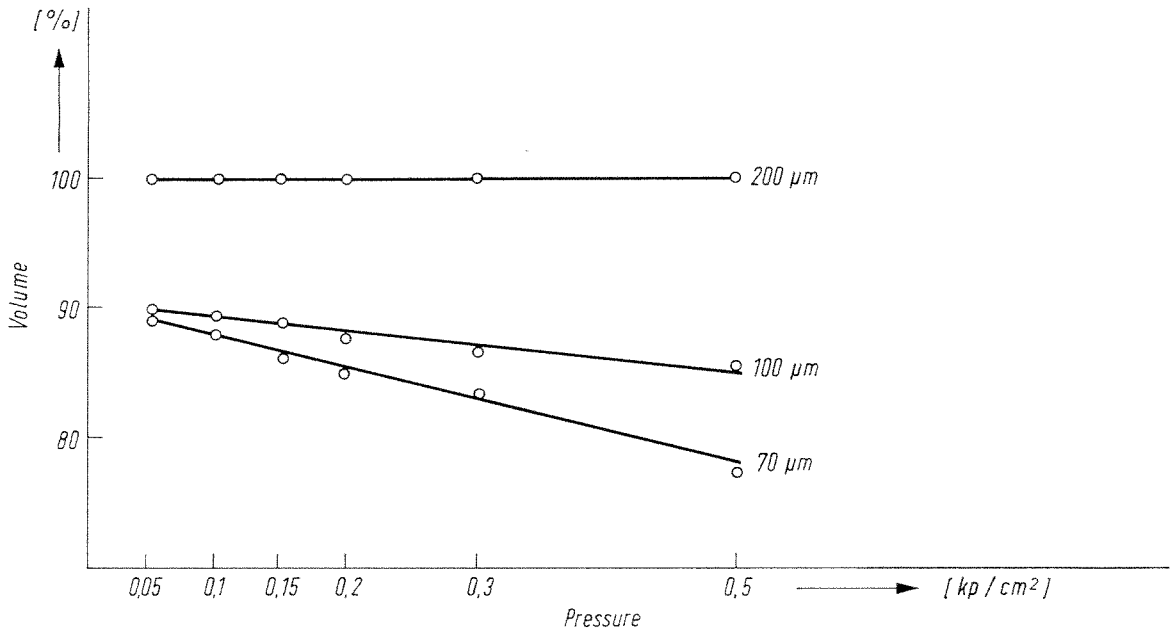

Fig. 1: The volume of 6-E polystyrene particles DVB measured with different suction pressures and capillaries.

Particle	6-E polystyrene particle DVB			Native human erythrocytes		Native rat erythrocytes	
Capillaries	70 µm	100 µm	200 µm	70 µm	100 µm	70 µm	100 µm
a) $V_1 = 10 \cdot \frac{R^4 \cdot X}{\rho \cdot D}$	611 ± 17	623 ± 13	689 ± 20	72 ± 5	73 ± 5	49 ± 3	50 ± 3
b) $V_2 = 10 \cdot \frac{R^4 \cdot X}{\rho \cdot D \cdot F}$	407 ± 11	415 ± 9	457 ± 13	72 ± 5	73 ± 5	49 ± 3	50 ± 3
c) V_E genuine volumes		551 ± 20			95 ± 6	66 ± 4	
d) K capillary factor	1.35	1.33	1.21	1.32	1.30	1.35	1.32

Tab. 1: Volumes of polystyrene particles, human and rat erythrocytes in μm^3:

a) Volumes obtained with the equation of Gutmann [7]
b) Volumes of a) – corrected for the form factor
F = 1.5 for polystyrene particles and
F = 1.0 for erythrocytes
c) Volumes obtained by the reference methods (see Methods)
d) Capillary factor $K = \frac{VE}{V_2}$.

impulse height and volume is observed sometimes due to shortening of the impulses. This dependence was tested for each capillary (Fig. 1). The mean particle volume for the 200 μm capillary is independent of the suction pressure from .05–.5 kp/cm². The volume for a 70 μm capillary at .5 kp/cm² suction pressure is about 20% lower than at .05 kp/cm². All measurements were therefore made at a suction pressure of .1 kp/cm². The electrical measurement of polystyrene particles yielded VDC skewed to the right, while those of erythrocytes yielded VDC with a Gaussian distribution as described previously [*9,18,19*]. The mean volumes of the polystyrene particles and the erythrocytes (Tab. 1) were calculated with the *Gutmann* equation [*7*] out of the mean volumes of each VDC.

Discussion

There is a clear discrepancy between the uncorrected mean particle volumes obtained by the reference methods and those obtained by electrical measurement and *Gutmann's* equation (Tab. 1). This is especially true for the polystyrene particles whose values are too high while those of human and rat erythrocytes are too low. Correct values are obtained with consideration of the following facts:

1) As previously mentioned, different form factors (F) are to be assigned to different types of particles depending on their form as they pass the capillary. These factors must be taken into account for the determination of their volumes by electronically as well as by particle calibrated measurements. For extremely elastic erythrocytes this factor is F = 1.0 [*1,10,18*], while for rigid particles, such as the polystyrene particles, it is F = 1.5 [*5,6*]. The form factors of other biological particles can be determined experimentally by photographing them during their passage through the capillary [*10*].

2) The equation used to calculate the particle volumes assumes a homogeneous electrical field over the whole length of the capillary, which for the Coulter capillaries with a length to diameter ratio of smaller than 1.0 (see methods 4.) is not the case [*6,10*]. That means a lowered volume value for the particles measured. A multiplicative factor which is specific for each capillary and corrects for the loss of volume is called capillary factor K and can be determined once by a single calibration using particles of known size. A mean capillary factor of 1.33 was obtained for the 70 μm and 100 μm capillaries and a K = 1.21 for the 200 μm capillary (Tab. 1).

Taking into account the form factor F and the capillary factor K the *Gutmann* equation [*7*] may be modified as follows:

$$V = 10 \frac{R^4 \cdot x \cdot K}{\rho \cdot D \cdot F} (\mu m^3) .$$

The electronic calibration of particle sizing apparatus basing on the principle employed in Coulter counters proves to be an accurate and practical mean for the determination of absolute particle volumes. The variation coefficient of the calibration factor D was 2.3% for 100 measurements over a period of 6 months. However, this calibration of the available devices is meaningful only in connexion with a central beam attachment and a multichannel analyser.

References: 1 Brecher, G., E. F. Jakobek and M. A. Schneidermann: Size Distribution of Erythrocytes. Ann. N. Y. Acad. Sci. *99,* 242 (1962). — 2 Coulter, W. H.: Instruction and Service Manual for the Model F Coulter Counter. — 3 Gabrieli, E. R. and M. Wertheimer: Standardisation of the Electronic Counter of Blood Cells. Am. J. Clin. Path. *36,* 277 (1961). — 4 Grant, J. L., M. C. Britton and Th. E. Kurtz: Measurement of Red Blood Cell Volume with the Electronic Cell Counter. Am. J. Clin. Path. *33,* 138 (1960). — 5 Gregg, E. C. and D. Steidley: Electrical Counting and Sizing of Mammalian Cells in Suspension. Biophys. J. *5,* 393 (1965). — 6 Grover, N. B., J. Naaman, S. Ben-Sasson and F. Doljanski: Electrical Sizing of Particles in Suspension. I/II. Biophys. J. *9,* 1398 (1969). — 7 Gutmann, J.: Elektronische Verfahren zur Ermittlung statistischer Maßzahlen einiger medizinisch wichtiger Daten. Elektromedizin *11,* 62 (1966). — 8 Hurley, J.: Sizing Particles with a Coulter-Counter. Biophys. J. *10,* 74 (1970). — 9 Kachel, V., H. Metzger und G. Ruhenstroth-Bauer: Der Einfluß der Partikeldurchtrittsbahn auf die Volumenverteilungskurven nach dem Coulter-Verfahren. Z. ges. exp. Med. *153,* 331 (1970). — 10 Kachel, V.: Methoden zur Analyse und Korrektur apparativ bedingter Meßfehler beim elektronischen Verfahren zur Teilchengrößenbestimmung nach Coulter. Dissertation TU Berlin 1972. — 11 Kelly, A. and R. E. Davis: Automatic Erythrocyte Volume Analysis: Price-Jones Curve Determination. Laboratory Practice *18,* 985 (1967). — 12 Kubitschek, H. E.: Electronic Measurement of Particle Size. Research *13,* 128 (1960). — 13 Lewis, S. M. and B. J. Burgess: A Stable Standard Suspension for Red Blood Counts. Laboratory Practice *15,* 305 (1966). — 14 Lusbaugh, C. C., J. A. Maddy and N. J. Basmann: Electronic Measurement of Cellular Volumes I: Calibration of the Apparatus. Blood *20,* 233 (1962). — 15 Mattern, C. F., F. S. Bracket and H. Byron: Determination of Number and Size of Particle by Electrical Gating: Blood Cells. J. Appl. Physiol. *10,* 56 (1957). — 16 Pfeiffer, G.: Elektronische Blutkörperchenzählung und Größenbestimmung. Z. med. Labortechnik *3,* 57 (1962). — 17 Scheidt, R. A. and W. J. Blake: Use of a Suspension of Latex Particles of Known Concentration for Monitoring the Coulter Counter. Am. J. Clin. Path. *35,* 293 (1961). — 18 Thom, R., A. Hampe und G. Sauerbrey: Die elektronische Volumenbestimmung von Blutkörperchen und ihre Fehlerquellen. Z. ges. exp. Med. *151,* 331 (1969). — 19 Valet, G., H. Metzger, V. Kachel und G. Ruhenstroth-Bauer: Der Nachweis verschiedener Erythrozytenpopulationen bei der Ratte. Blut *14,* 42 (1972).

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